COMPENSATION OF LIVER PROTEIN SYNTHESIS IN TEMPERATURE-ACCLIMATED TOADFISH, OPSANUS TAU

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In the process of temperature acclimation poikilotherms adjust their metabolic rates in order to maintain physiological activity at a more nearly constant level over a range of environmental temperatures. Thus, they attain a measure of independence of temperature (Prosser and Brown, 1961). A review of the earlier literature dealing with this phenomenon and discussion of its significance in ecology and evolution has been given by Bullock (1955). More recently, the subject has been reviewed in several symposia (Prosser, 1958; 1967; Troshin, 1967). Most studies have dealt with temperature acclimation in terms of physiological (heart rate, oxygen consumption) or behavioral parameters; it is only in the last few years that methods have become available to attempt an approach to the molecular basis of the phenomenon.

Several recent studies have presented evidence for changes in level of enzymes in response to acclimation. Freed (1965) demonstrated an increase in cytochrome oxidase activity in goldfish muscle as measured at 15° after the fish had been acclimated to 5°; decreased activity resulted from warm acclimation (25°). Similarly, increased 6-phosphogluconic dehydrogenase activity is found in gills of cold-acclimated carp (Ekberg, 1962). Hochachka and Hayes (1962) showed greater utilization of the pentose shunt in cold-acclimated trout. Changes in the lactic dehydrogenase isozyme system have been found to accompany thermal compensations in goldfish (Hochachka, 1965). Because of the probability that these activity increases are due to increased levels of enzyme protein, a study was begun on the effect of low temperature acclimation on protein synthetic capacity in fish. The toadfish was chosen as the experimental animal because of its ready adaptability to laboratory conditions, and its wide range of natural habitat (Bigelow and Schroeder, 1953; Henshall, 1891), suggesting a high degree of temperature adaptability. Studies were concentrated on the liver where (in the case of rat) a good deal has been learned about the mechanism of protein synthesis. Of equal importance to the question of the mechanism of temperature acclimation is the possibility that the phenomenon can be used to elucidate sites of regulation in the protein synthetic system *per se*. A preliminary report of this work has appeared (Haschemeyer, 1967).

Materials and Methods

Animals

Adult toadfish, 200–300 g., were obtained from the Supply Department at the Marine Biological Laboratory, Woods Hole, and included both sexes. In the first series of experiments the fish were kept in running sea water aquaria at the Marine Biological Laboratory. With the available refrigeration system it was possible to keep groups of up to six fish at a temperature of 10° ± 1° in running sea water during the period of low temperature acclimation. Control fish were kept at 20–22°, the laboratory temperature of the sea water supply at Woods Hole at that time. In the second series of experiments the fish were maintained in aerated static aquaria prepared with a synthetic sea salt mixture (Rila Products, Teaneck, N. J.). For low temperature acclimation the fish were kept in a 25-gal. Instant Ocean aquarium (Aquarium Systems, Inc., Wickliffe, Ohio) with temperature control at 10° ± 0.5°. Control fish were kept at room temperature, 22–24°. The toadfish were fed small *Fundulus heteroclitus* to appetite.

Measurement of in vivo protein synthesis

For determination of incorporation of C^{14} amino acids into liver protein, a rapid arterial injection route was used, as described by Cooperstein and Lazarow (1964). In cases where the incubation temperature for the measurement differed from the acclimation temperature, the fish was transferred to the new temperature ½–1 hr. before injection. Each fish was wrapped in cheesecloth with its tail brought around to the right of the body, and a hole was cut in the cloth over the left gill. The skin on either side of the operculum was clipped in order to expose the gill arches and 0.2 ml. of a solution containing 10 μc. of C^{14} amino acids was injected through a 25-gauge, ⅝″ needle into the branchial artery of the fourth gill arch. The isotope solution was prepared from a mixture of 15 purified C^{14}-L-amino acids (New England Nuclear Corp.) of high specific activity (1 mc./mg.); the commercial solution in 0.1 N HCl was neutralized, buffered with 0.5 *M* Tris, pH 7.4, and brought to 1% NaCl. After injection the gill flap was held closed for about 5 seconds to aid clotting at the needle hole, and the fish was returned to a pail of sea water for the incubation period. The entire procedure took about 2 minutes; the fish started swimming as soon as returned to water and showed no ill effects. After time intervals of 5 min. to 30 min. the fish was stunned by a blow to the head, and the liver quickly excised and transferred to cold Medium A (0.25 *M* sucrose, 0.05 *M* Tris, pH 7.4, 0.025 *M* KCl, 0.01 *M* $MgCl_2$). In some cases to determine free radioactivity in the blood as a measure of the effectiveness of the injection, approximately 0.7 ml. of blood was collected by syringe from the heart cavity and added to 0.1 ml. of 0.1 *M* ethylenediamine tetraacetate, K^+, pH 8.0.

Each liver was accurately wet-weighed, minced and homogenized in 3 volumes cold Medium A in a Sorvall Omni-mixer at half of full speed. For the second series of experiments in which biochemical analyses were made, the livers were homogenized in 3 volumes of ice-cold distilled water. The homogenates were analyzed for C^{14} incorporation into protein using a filter-paper disc method, based on the method of Bollum (1959). For each homogenate 100-μl. aliquots were

pipetted onto several discs (Whatman 3MM filter paper, 2.3 cm. diameter). After a few seconds to allow the aliquot to soak in, all discs were dropped into a washing solution of ice-cold 10% trichloroacetic acid. The amount of washing solution in this and subsequent steps was about 5–10 ml. per disc; the time for each step was about 15 min., during which the solution and discs were swirled occasionally. A second 10% trichloroacetic acid wash was done, then five washes in cold 3% perchloric acid, two in cold 95% ethanol, and two in absolute ether. The discs were air-dried, placed in glass vials and counted in a Packard Tri-Carb liquid scintillation counter using a toluene solution of 4 g./l. 2,5-diphenyloxazole and 0.5 g./l. 1,4-bis-2-(5-phenyloxazole)-benzene.

Free radioactivity in the liver homogenates was determined as follows: 1.00 ml. of the homogenate in a clinical centrifuge tube was treated with 1.00 ml. of cold 10% trichloroacetic acid and kept in ice for 15 min. The protein-nucleic acid precipitate was spun down, and 100 μl. of the clear supernatant were pipetted into a glass vial with 10 ml. of Bray's scintillation solution (Bray, 1960) and counted in the Packard scintillation counter. Values for TCA-soluble radioactivity therefore refer to homogenate which has been diluted by a factor of two, whereas the values for protein (TCA-precipitable) radioactivity refer to the undiluted homogenate. Free radioactivity in the blood was determined as for the liver homogenate except smaller volumes were used.

Biochemical analyses

DNA, RNA, and protein analyses following Schneider (1945) were made at three concentrations of the liver homogenates prepared in distilled water. Three clinical centrifuge tubes were prepared containing 0.25, 0.50 and 1.00 ml. of the homogenate. The first two were brought to 1.00 ml. with distilled water and all were treated with 1.00 ml. of cold 10% perchloric acid to precipitate proteins and nucleic acids. After the precipitate was washed with cold 10% perchloric acid and twice with cold 95% ethanol, the nucleic acids were hydrolyzed in 3 ml. 5% perchloric acid at 90° for 15 min. The supernatant was removed and analyzed for total nucleic acids by ultraviolet absorption at 260 mμ, for RNA with orcinol and for DNA with diphenylamine. The protein precipitate was collected on a pre-weighed Millipore filter, air-dried, and weighed. The diphenylamine reaction gave $OD_{600} = 0.364$/mg. Na DNA, based on standard solutions of calf thymus DNA (General Biochemicals). The DNA content of this preparation (as Na DNA, residue weight 331) was found to be 0.66 mg. per mg. of weighed material, as determined from OD_{260} of an acid hydrolysate; the conversion factor $OD_{260} = 30$ for 1 mg./ml. hydrolyzed Na DNA was calculated from extinction coefficients of isolated nucleosides (Beaven *et al.*, 1955). Recovery in the sample preparation was 75% for calf thymus DNA, and DNA values for the liver homogenates were corrected accordingly. Results are based on the slope obtained in a plot of OD_{600} *vs.* concentration of homogenate. A similar plot of OD_{260} for the hot perchloric acid supernatant *vs.* concentration yielded values for total nucleic acid concentration in the homogenate. A standard nucleoprotein consisting of purified *E. coli* K 12 ribosomes, prepared according to Nirenberg (1964), was tested in the procedure. Recovery of the ribosomal RNA was

about 75% after precipitation, washing and hydrolysis, compared with direct acid hydrolysis. Total nucleic acid as mg./ml. of liver homogenate was estimated on the basis of this figure and the conversion factor above, and the DNA content was subtracted to obtain RNA. The results in Table III are expressed in mg. per g. wet weight of liver. Measurement of RNA by the orcinol procedure was unsatisfactory due to apparent interference by a liver component which contributed as much as one-half of the total orcinol reading.

Free amino acid analyses were made by the method of Spies (1952) although modification was required because of interfering materials in the whole liver homogenate. The homogenate was first spun at 15,000 *g* for 10 min. in a Sorvall refrigerated centrifuge to remove large particles and debris and then at 105,000 *g* for 3 hr. in a Spinco L2 centrifuge (at 4°) to remove glycogen and ribosomes. The supernatant was carefully withdrawn by pipette, avoiding the lipid layer; proteins were precipitated by addition of 70% perchloric acid to a final concentration of 5%. The precipitate was spun down in a clinical centrifuge and the clear supernatant was neutralized with NaOH and analyzed at two con-

TABLE I

Effect of low temperature acclimation (10°) on protein synthetic capacity of toadfish liver measured in vivo (running sea water aquaria). Incubation time after arterial injection = 15–30 min. Data expressed as cpm in trichloroacetic acid precipitate (on filter paper disc) divided by cpm in trichloroacetic acid-soluble supernatant

Acclimation period Days at 10°	Number of animals*	Temperature of measurement	Incorporation into protein compared to available free radioactivity in liver
0	9	20–22°	0.24 ± 0.15 (S.D.)
3	4	20–22°	0.27 ± 0.15
3	4	10°	0.09 ± 0.04
7	3	20–22°	0.48 ± 0.11

* Average body weight = 240 g.

centrations for the 230 mμ absorption produced by Cu^{++}-amino acid complexes. Each solution was read against a blank consisting of all ingredients except the $CuCl_2$ reagent. All optical density readings were made with a Beckman DU spectrophotometer equipped with a Gilford Model 2000 photomultiplier unit and recorder. A standard solution of L-alanine (Schwarz Laboratories, Inc.) gave a value of $OD_{230} = 3.2$ for a concentration of 1 μm./ml. amino acid in the final solution.

RESULTS

Table I shows the results of the first series of experiments on the effect of cold acclimation on incorporation of radioactive amino acids into liver protein *in vivo*. The nine control animals included fed and starved (3–7 day) individuals: there were not enough animals to establish a correlation between nutrition and protein synthetic rate. The fish kept at 10° did not eat during this period, although minnows were available in the tank. As shown, three days at 10°

produced no change in liver protein synthesis, when the fish were returned to 20–22° for measurement. When the measurement was made at 10°, the fish showed a low rate of synthesis, as expected for a drop of 10°, in the absence of compensation due to acclimation. The approximate Q_{10} is 2.5, based on the values in Table I and taking the higher temperature as 21°. Values of free radioactivity in the liver supernatants ranged from 500 to 3500 cpm. In two comparable groups the average for fish incubated at 10° was slightly lower (750 cpm) than that for fish incubated at 20° (1050 cpm). Measurements of free radioactivity in the blood correlated well with that in liver for each individual; either of these quantities thus provided a measure of the effectiveness of the injection. To eliminate this factor all the data have been expressed as the ratio of cpm in protein to cpm in the trichloroacetic acid-soluble phase of the liver homogenate. The ratio did not depend on time of incubation between 15 and 30 min., although small changes would have been obscured by the large variation among individuals. Shorter incubations did show time dependence and are not included in Table I. The large scatter in the final data, as indicated by the reported standard deviations, must be

TABLE II

Effect of low temperature acclimation (10°) on protein synthetic capacity of toadfish liver measured in vivo (static artificial aquaria). Incubation temperature = 23°; time = 20–30 min.

Expt. no.	Days at 10°	Nutritional state	Number of animals	Incorporation into protein compared to available free radioactivity
1	0	Starved 5 days	4	0.21 ± 0.11 (S.D.)
2	0	Fed	3	0.23 ± 0.16
3	3	Starved 4 days	3	0.13 ± 0.03
4	14	Starved 14 days	4	0.37 ± 0.12
5	14	Fed	4	0.30 ± 0.07

attributed to population variability of unknown origin. Hormonal influences on liver metabolism following the stress of handling and injection may play a role. Adrenal and pituitary hormones have been shown to influence levels of C^{14} leucine incorporation into protein in rat liver following a single intraperitoneal injection of the isotope (Reid *et al.*, 1956).

After seven days at 10°, as shown in Table I, an increase in protein synthesis is observed upon measurement at 20°. It must be noted that the number of animals is small and the standard deviation in all these measurements rather large; the absolute values must be viewed accordingly. However, analysis by the standard t-test indicated the difference between the 10°-acclimated and control groups to be significant at $P = 0.05$. Thus, the results strongly suggest that liver protein synthesis in toadfish exhibits at least a partial compensation in Precht's (1958) terminology.

The results for *in vivo* protein synthesis in the second series of experiments are given in Table II. In this series more attention was given to nutrition, although again no reliable correlation between feeding and protein synthesis could be established. Fish labelled "fed" had taken minnows one or two days before the experiment; remains were found in the gut at autopsy. The fish kept at 10°

showed little interest in the minnows in the tank, but even after two weeks the toadfish showed no significant weight loss or other apparent sign of starvation. For Expt. 5 the fish were hand-fed by holding minnows in front of them and tapping the mouth until they snapped angrily. The fish were also observed carefully for behavioral changes during 10° acclimation. Unfortunately the toadfish is by nature a sluggish bottom dweller, and the specimens used here conformed to this description equally well at 10° and 20°. Respiratory movements averaged about 8 per min. at 10° and did not change over the two-week acclimation period. The only difference noted was that some of the two-week acclimated fish, after having been warmed to 20°, were hyperactive and difficult to handle during the injection procedure. Effects of low temperature acclimation on oxygen consumption in this species have not been reported, although it would be particularly interesting in view of the unusually high skin respiration of toadfish which permits them to survive 1–2 days out of water (Schwartz and Robinson, 1963).

As indicated in Table II, the two groups of control (23°) fish, fed and 5-day starved, gave comparable values for liver protein synthesis. The fact that all values in the second series are less than in the first is due primarily to the lower efficiency for counting filters of the scintillation counter used for the second series. This, of course, does not affect the results on a comparative basis. Other differences in the second series were the use of static aquaria, and the slightly larger size of the fish collected later in the season. Females in this group had large well-developed eggs. In order to test for a possible effect of temperature shock as a result of transfer from 10° to over 20° immediately before the experiment, a group of fish was again assayed after 3 days at 10°. The average value of 0.13 is low compared to the controls; it is not certain what significance this may have, particularly since it was not observed in the first series. In any case, the sudden transfer of the fish from 10° to 23° does not produce by itself the increase of protein synthesis found after the longer periods at 10°. Expts. 4 and 5 in Table II show the effect of 14 days of 10° acclimation on liver protein synthesis. Comparison of the average of Expts. 4 and 5 with the average of 1, 2 and 3 yields an increased protein synthesis of about 75% as a result of low temperature acclimation (significant at $P = 0.2$ by the t-test). This is in good agreement with the results of Table I.

Biochemical analyses were also performed in the second series to determine if any major alterations of the cellular constituents involved in protein synthesis occurred as a result of 10° acclimation. Of particular importance is the free amino acid pool of liver because the amount of incorporation of the injected isotope will depend on the specific activity of the pool as well as on the rate of protein synthesis. If the pool size does not vary greatly, then the present method of measurement, based simply on cpm per unit volume of homogenate, is justified. On the other hand, if the amino acid pool in cold-acclimated fish is low, then the specific activity will be higher for the same amount of isotope, and incorporation of label into protein will be increased accordingly (this assumes that the rate is not directly dependent on pool concentrations, in which case, the effect would cancel out). As shown in Table III, although there is some decrease in free amino acid concentration in the cold-acclimated fish (Expts. 4 and 5), it is not sufficient to account for the 75% increase in incorporation into protein

shown in Table II. In fact, the observation that fish at 10° for 3 days (Expt. 3) show the same low pool value suggests that the decrease may be related to the low temperature treatment but not to the acclimation phenomenon. The present values for free amino acid concentration in a fish liver are similar to those reported for rat liver. The sum of the isolated amino acids of rat liver (one day starved or protein-fed animals) reported by Wiss (see Tarver, 1963) amounts to 24 μmoles/g. liver.

Individual liver-to-body-weight ratios generally showed the expected increase with body weight (Robinson *et al.*, 1960); average ratios in the cold acclimated fish are slightly lower than controls, probably because the fish did not eat during most of the period. This is also indicated by the slight increase of DNA per gram of tissue. Protein yields, however, did not change. Although no analysis was done for lipid and glycogen, it was noted during the centrifugation steps used in preparation for amino acid analysis that the lipid layer formed in the 15,000 *g*

TABLE III

Biochemical analyses for livers of control and cold-acclimated toadfish (incorporation data in Table II)

Expt. no.	Body wgt. average	% Liver	mg./g. tissue (wet)			μm./g. tissue
		body	DNA	RNA	Protein	Free amino acids
1	241	2.8	2.36 ±0.27	3.85 ±1.40	135 ±15	29 ±3
2	314	4.0	2.28 ±0.30	4.15 ±1.30	112 ±6	26 ±3
3	250	3.7	2.26 ±0.25	4.05 ±2.00	116 ±9	23 ±2
4	279	3.1	2.84 ±0.19	5.45 ±1.95	110 ±7	23 ±2
5	260	2.8	2.48 ±0.25	3.80 ±1.95	133 ±17	21 ±2

centrifugation was almost entirely lacking in cold-acclimated fish and the glycogen pellet obtained at 105,000 *g* was reduced in size. This may account for the slight loss in liver mass in the cold-acclimated animals.

The values obtained for total RNA per g. liver showed wide variation, as indicated by the large standard deviation. Examination of the individual data revealed that values clustered around two levels; about 6 mg./g. and about 2 mg./g. Averaging of the high and low values separately yielded values of 5.70 ± 0.70 mg./g. for 11 fish and 2.15 ± 0.20 mg./g. for 7 fish. These are both low compared with values obtained by the same method for rat liver (Haschemeyer and Gross, 1967) of about 8 mg./g. in total homogenates. The finding of the higher RNA content for the seven fish which contained eggs suggested a correlation of liver RNA content with the sex of the animal. The levels of all tissue constituents measured differ appreciably from those reported for goldfish liver (Das, 1967), when compared in the same units. In that study, however, the fish were well-fed, with resultant hypertrophy of the liver in the low temperature group and concomitant increase of protein and decrease of

DNA in relation to tissue mass. In spite of the higher rate of protein synthesis, such hypertrophy was not observed in the toadfish liver, probably because the fish ate little on their own initiative during the low temperature period.

Discussion

The purpose of these experiments was to determine what type of compensation might occur in liver protein synthesis of a marine fish, following varying periods at a temperature at the lower end of the animal's normal environmental range. Protein synthesis was assessed in terms of the incorporation of C^{14} amino acids into protein compared to the available free radioactivity in the liver. The procedure involving injection into the arterial blood system permitted rapid uptake of labelled amino acids by the liver; thus incorporation into protein could be measured after short intervals, before degradation or export would be expected to significantly affect the levels of labelled proteins. Rapid pulse measurements are well advised in view of the short half-lives of some liver proteins (in rat), particularly inducible enzymes; tryptophan pyrrolase (peroxidase), for example, is degraded with a half-life of about 2.3 hr. (Feigelson *et al.*, 1959). Other proteins are relatively long-lived; ribosomal protein probably has a half-life of five days, like that of liver ribosomal RNA (Loeb *et al.*, 1965).

The data presented here establish that on the average, toadfish acclimated under laboratory conditions to a temperature of 10° exhibit an increase in liver protein synthesis of about 75% over control fish acclimated to 20–23°, when both are measured at the higher temperature. The biochemical analyses indicated little alteration in tissue components of liver involved in protein synthesis, under the acclimation conditions used in these experiments. Most important, no significant change in free amino acid pools occurred, which would produce some uncertainty in the use of incorporation data as a measure of protein synthesis. Although Das and Prosser (1967), in a study of goldfish acclimation reported during the course of the present work, were unable to establish a significant effect in liver in short-time experiments, this may have been due to the use of intraperitoneal injection. They found, however, that accumulation of label in protein over long periods was increased in cold-acclimated fish, consistent with a greater protein synthetic rate.

The present results support the hypothesis that a control in the protein synthetic pathway may provide a common basis for increased levels of enzymes in respiratory metabolism and other essential pathways responsible for physiological adaptation to low temperatures. Such a change must of course be accompanied by associated changes in degradation rates or rates of protein export from the liver to arrive at a new equilibrium level of proteins. Although a common site for control is suggested by the magnitude of the effect, this does not mean that all proteins are necessarily equally affected; for example, an increase in rate at a step common to all proteins will not affect proteins whose synthesis is rate-limited at another step. The possibility of selective control has been suggested by the finding that different codons are used in the insertion of leucine (Weisblum *et al.*, 1965) and arginine (Weisblum *et al.*, 1967) into the α chain of rabbit hemoglobin. In the case of leucine it was shown by *in vitro* protein

synthesis with *E. coli* transfer RNA that a minor species of leucine-accepted tRNA was required to insert leucine into a particular site on the α chain. Thus, the level of a "rare" transfer RNA or its associated aminoacyl-tRNA synthetase may limit the synthesis of some proteins, even during a state of generally increased protein synthesis. Another way in which proteins may be selectively controlled results from special requirements for release from the messenger RNA-ribosome complex or their influence on polyribosome activity. It has been suggested that in hemoglobin the dependence of globin synthesis upon the availability of the prosthetic group is due to a control over release from the protein synthetic site (Gribble and Schwartz, 1965). Another study shows increased size and stability of reticulocyte polyribosomes in response to hemin addition (Grayzel *et al.*, 1966).

Thus, the increased protein synthetic rate in toadfish liver provides a simple mechanism for obtaining increased levels of many proteins, without requiring a specific derepression of genes, as in the Jacob-Monod model (1961) of bacterial enzyme induction. Although the present results refer only to liver, it is quite likely that other tissues exhibit the same phenomenon. Das and Prosser (1967) found evidence for temperature compensation of protein synthesis in gill and muscle tissue of goldfish. Mews (1957) and Jankowsky (1960) have reported increased protein synthesis during cold adaptation of frog skeletal muscle. In order to investigate the possibility of a common translational-type control over protein synthesis, subject to variation with acclimation temperature, we have turned to the study of protein synthesis and the components of the protein synthetic system *in vitro*. These studies will be reported in a separate communication.

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Summary

Liver protein synthesis was measured with a rapid arterial injection procedure in control (20–23°) and cold-acclimated (10°) toadfish. The results were expressed as the cpm of radioactive amino acids incorporated into protein compared to cpm of free radioactivity in the liver homogenate, to correct for variability in injections and in uptake of amino acids by the liver. The results show that, when measured at 20–23°, the 10° acclimated fish possess liver protein synthetic capacity about 75% greater than fish maintained at the higher temperature. Two series of experiments, in which fish were maintained in running sea water aquaria or in static artificial sea water aquaria, gave comparable results. The livers were analyzed for DNA, RNA, protein and free amino acids. The levels of these constituents resembled those in mammalian liver with the exception of RNA, which was lower. Under the conditions of these experiments no significant changes were observed in the constituents measured as a result of two-week cold acclimation.

The possibility of a common translational-type control over protein synthesis to account for increased enzyme levels in cold temperature acclimation is discussed.

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